

# Osteoarthritis and Cartilage



## The contribution of collagen fibers to the mechanical compressive properties of the temporomandibular joint disc



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### SUMMARY

**Objective:** The Temporomandibular Joint (TMJ) disc is a fibrocartilaginous structure located between the mandibular condyle and the temporal bone, facilitating smooth movements of the jaw. The load-bearing properties of its anisotropic collagenous network have been well characterized under tensile loading conditions. However, recently it has also been speculated that the collagen fibers may contribute dominantly in reinforcing the disc under compression. Therefore, in this study, the structural-functional role of collagen fibers in mechanical compressive properties of TMJ disc was investigated.

**Design:** Intact porcine TMJ discs were enzymatically digested with collagenase to disrupt the collagenous network of the cartilage. The digested and non-digested articular discs were analyzed mechanically, biochemically and histologically in five various regions. These tests included: (1) cyclic compression tests, (2) biochemical quantification of collagen and glycosaminoglycan (GAG) content and (3) visualization of collagen fibers' alignment by polarized light microscopy (PLM).

**Results:** The instantaneous compressive moduli of the articular discs were reduced by as much as 50–90% depending on the region after the collagenase treatment. The energy dissipation properties of the digested discs showed a similar tendency. Biochemical analysis of the digested samples demonstrated an average of 14% and 35% loss in collagen and GAG, respectively. Despite the low reduction of collagen content the PLM images showed considerable perturbation of the collagenous network of the TMJ disc.

**Conclusions:** The results indicated that even mild disruption of collagen fibers can lead to substantial mechanical softening of TMJ disc undermining its reinforcement and mechanical stability under compression.

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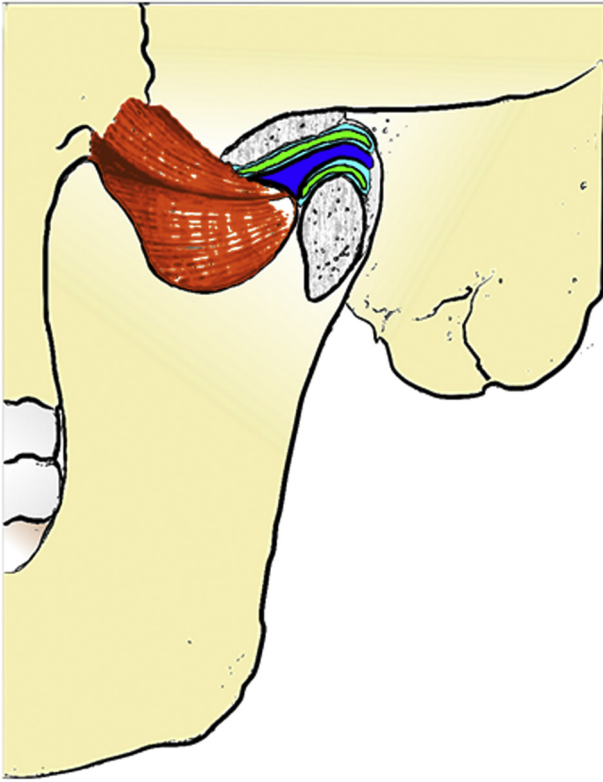
### Introduction

The disc of the temporomandibular joint (TMJ) is a robust fibrocartilaginous structure located between the articulating surfaces of the mandibular condyle and temporal bone (Fig. 1). It facilitates smooth jaw movement by increasing congruity, mediating forces, and absorbing impact loads exerted by the articular

bones during everyday activities such as mastication, talking and yawning<sup>1,2</sup>. Most epidemiological studies have reported that 20–25% of the population exhibits one or more symptoms of TMJ disorder (TMD)<sup>3</sup>. Of the three TMJ components, the disc is of particular interest since approximately 70% of patients with TMD suffer from malpositioning of the disc, known as internal derangement<sup>4</sup>. Despite the elusive mechanism underlying disease progression, internal derangement appears to be highly correlated with TMJ osteoarthritis (OA) as an accompanying sign or a subsequent factor in a later stage<sup>5</sup>. Due to a poor understanding of TMD etiology, options for treatment of severely damaged disc are restricted to its resection<sup>6,7</sup>. However, since the disc is a crucial functional component of TMJ, its removal can lead to further pain and dysfunction, and eventually degeneration of the whole joint<sup>7</sup>. The presence of a functionally reliable replacement could be helpful for

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**Fig. 1.** Schematic view of the TMJ. The TMJ disc (blue), and the articular cartilage of the condyle and mandibular fossa (green) are shown from sagittal view.

treatment, but such has not been engineered yet. To reach that aim the structural-functional relationship of the native TMJ disc needs to be established.

The human TMJ disc is composed of two principal components: (1) a solid extracellular matrix, occupied predominantly by a highly organized collagenous network, and a sparse amount of GAG; and (2) a movable interstitial fluid containing water<sup>8,9</sup>. In contrast to other joints 70–80% of the dry weight of the TMJ disc consists of collagen type I, while GAG constitute only 0.6–10% of it<sup>10</sup>. In the intermediate zone (IZ), the collagen fibers are aligned anteroposteriorly, while in the periphery they show a ring-like orientation, merging with mediolaterally aligned fibers in the posterior and anterior bands<sup>11</sup>. It is commonly assumed that the highly negatively charged GAG and other proteoglycans (PGs) are considered to create an intra-tissue osmotic swelling pressure. Herewith, they respond to compression-induced hydrostatic pressures to spread the force away from directly loaded areas<sup>12,13</sup>. This is performed by interaction with the highly organized region-specific fibrillar collagen of the extracellular matrix of the TMJ disc<sup>11,14</sup>. These features provide the disc with anisotropic and heterogeneous properties that reinforce its structure and maintain its integrity under various types of loading. In a TMJ disc, which is pathologically degenerated by extrinsic factors (e.g., abnormal/repetitive mechanical stress) or/and intrinsic factors (e.g., enzymatic-induced degradation), collagenous network loses its dense integrity. This eventually leads to a disruption of the solid-fluid load-bearing continuum in the cartilage<sup>4,5,15</sup>.

Generally, the mechanical compressive properties have been attributed to the GAG content<sup>16,17</sup>, while tensile mechanical properties have been related to the amount and organization of collagen fibers<sup>18,19</sup>. However, Willard *et al.*<sup>20</sup> recently indicated that the regional mechanical compressive properties of the disc have a stronger correlation with the associated collagen density and

structure than with the GAG content. Despite few studies<sup>8,17,21,22</sup> describing region-dependent mechanical compressive properties of the excised TMJ disc samples, the question still remains how collagen content and structure correspond to regional variations in compressive stiffness of the disc.

Therefore, we measured the regional mechanical compressive properties of TMJ discs before and after enzymatic digestion of their collagen fibers. This approach helps us to evaluate the contribution of collagen fibers in providing reinforcement and stability to the extracellular matrix of TMJ disc as a whole and in relation to their region-specific morphology.

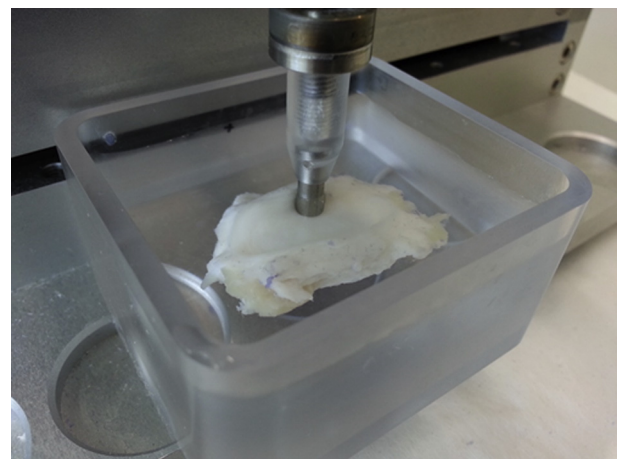
## Methods

### Sample preparation

Five young porcine heads were obtained from a local slaughterhouse and processed immediately after sacrifice. First, the TMJ discs and condylar head were dissected *en bloc* and then the discs were carefully isolated by removing all bony parts and peripheral soft tissue. All discs were inspected visually and no gross abnormalities were observed. After isolation, the TMJ discs were washed in phosphate buffered saline (PBS), wrapped in gauze soaked with solution of PBS and a mixture of protease inhibitors (Roche Diagnostics, Germany), and then stored at  $-20^{\circ}\text{C}$  at which temperature the biomechanical properties of the disc are not affected<sup>23</sup>.

### Experimental apparatus

The mechanical loading experiment was performed by a custom-made instrument (Fig. 2) capable of generating sinusoidal displacement with a resolution of  $0.1\ \mu\text{m}$  at a rate of maximally 30 Hz as described previously by Berendsen *et al.*<sup>24</sup>. The instrument consists of a chamber and two cylindrical stainless steel indenters with diameter of 4 mm. The bottom indenter was fixed to the surface of the chamber and it was aligned with the top indenter whose displacement was controlled by a custom-made software (implemented in LabVIEW 8.2, National Instruments, Austin TX). The samples were placed on the lower indenter, inside the PBS-filled chamber, and the rigid upper indenter was used to apply cyclic compressive displacement. The normal reaction force exerted to the top indenter was measured by a 25 N load cell (Honeywell Model 11, Honeywell, Golden Valley MN). The signal of the load cell was amplified by a bridge amplifier (HBM K10, HBM, Darmstadt,



**Fig. 2.** Indentation test on TMJ disc of porcine sample by the custom-made indentation device.

Germany) and registered by the same application that also controlled the indenter. Displacement and reaction force was registered simultaneously with intervals of 16 ms.

#### Mechanical loading experiment

To determine the regional differences in mechanical compressive properties of the TMJ disc, all samples were cyclically loaded at five different locations namely; anterior band (AB), posterior band (PB), intermediate zone medial (IZM), intermediate zone lateral (IZL) and intermediate zone central (IZC). The discs were tested intact in different regions, thereby maintaining their integrity and confinement.

Prior to the mechanical loading experiment, the frozen discs were immersed in PBS solution and allowed to thaw at room temperature for 1 h. Then, the inferior surface of the discs was glued to the bottom indenter using a thin layer of cyanoacrylate (Histoacryl, Braun Surgical S.A., Rubi, Spain), after which, the chamber was filled with PBS solution and samples were allowed to equilibrate for 5 min. To maintain a proper contact between the top indenter and surface of the discs, a tare load of 0.02N was applied to each sample followed by 5 min of relaxation time. Then, the thickness of the sample was measured as the platen-to-platen distance using a digital caliper. After a steady state contact was obtained, a 3-minute preconditioning test of 10% strain amplitude with 1 Hz frequency was conducted for all samples to create a uniform loading history. Accordingly, samples were allowed a further 5 min of recovery. Thereafter, the discs underwent a dynamic test including 20 cycles of same loading profile carried out in the preconditioning test (10% strain at 1 Hz). Following completion of the test at one location, the disc was carefully detached from the bottom indenter, glued at another location where it was tested with the same protocol. The 10% compressive strain amplitude was determined according to the amount of joint space reduction during maximum clenching (5–10%)<sup>25</sup>. Additionally, a loading frequency of 1 Hz was chosen to simulate the average chewing frequency (0.5–1.5 Hz) in human<sup>25</sup>. Although porcine chewing is faster (2–3 Hz)<sup>26</sup>, previous studies<sup>21,25</sup> reported mechanical data of porcine disc at 1 Hz which can be used for data comparison.

Deformation of the disc (strain) was determined by the ratio of the displacement of the upper indenter to the initial thickness of the sample located between the platens. The stress was defined as the ratio between the reaction force and cross-sectional area of the indenter. Subsequently, to characterize the basic mechanical compressive properties of the disc, the instantaneous modulus as the maximum stress of the first cycle relative to the respective strain and the maximum hysteresis as the enclosed area between the first stress-strain loop were calculated.

#### Enzymatic digestion

To assess the role of collagen fibers in compressive stiffness of the disc, samples were divided into two groups: control (PBS) (left discs,  $n = 5$ ) and treated (collagenase) (right discs,  $n = 5$ ). All samples were mechanically tested before and after incubation, thus serving as their own controls. Following the first mechanical test, samples were equilibrated in PBS for at least 30 min at room temperature. Then, the right side discs were submersed in 25 mL of PBS solution containing 7.5 mg of collagenase type II (100 U/mL, Worthington Inc., Lakewood, NJ) and incubated under gentle agitation at 37°C for 16 h. As a control group, the left side discs were incubated in PBS solution under the same condition. After the incubation, all samples were washed three times with saline buffer and then submersed in fresh PBS for 30 min at room temperature.

Subsequently, all samples underwent the post-incubation mechanical test using the same loading protocol as described above.

#### Biochemical analysis

After the second mechanical test, the samples were equilibrated in PBS for 30 min at room temperature. To determine the regional collagen and GAG content of the samples, a 4 mm disposable dermal punch (Miltex Instruments, Lake Success, NY) was used to excise specimens from the five mechanically loaded regions of the TMJ discs. The residual of the discs were fixed overnight in a paraformaldehyde solution (4%) at 4°C, subsequently placed in fresh PBS and stored for polarized light microscopy (PLM) analysis. Extracted specimens were lyophilized overnight and weighted dry. Following a 16-hours papain (Sigma, St. Louis, MO) digestion, the total hydroxyproline and GAG content were determined by using the protocol described by Ghazanfari *et al.*<sup>27</sup>. The GAG content was measured by 1–9-dimethylmethylene blue dye-binding assay using chondroitin sulfate from shark cartilage (Sigma, St. Louis, MO) as the standard. The hydroxyproline, which served as a measure of total collagen content, was assessed colorimetrically following alkaline hydrolyzes of the digest and its subsequent reaction with chloramine-T and dimethylaminobenzaldehyde.

#### Microscopy

The effect of collagenase digestion on the spatial alignment of collagen fibers was evaluated by visualizing unstained histological sections under PLM (Leica DM EP, Germany). Therefore, the discs were cut into three parts (central, lateral and medial) and each part was further sliced into two parts, which were subsequently embedded in Tissue-Tek (Sakura Finetek, Netherlands) and cryosectioned at 10 µm along the superoinferior or sagittal plane. The sections were mounted on silane-coated glass slides and allowed to dry. Thereafter, they were coverslipped with Entellan (Merck, Germany) and examined by PLM.

#### Statistical analysis

To determine whether there were significant biological variations in mechanical properties between the left and right side samples within a single region we performed a paired student's *t*-test on the mechanical data obtained before PBS or collagenase incubation. Since there were no significant differences between mechanical properties of the left and right side samples, we continued further analysis by only considering the mechanical data obtained from the control (PBS) and treated (collagenase) groups.

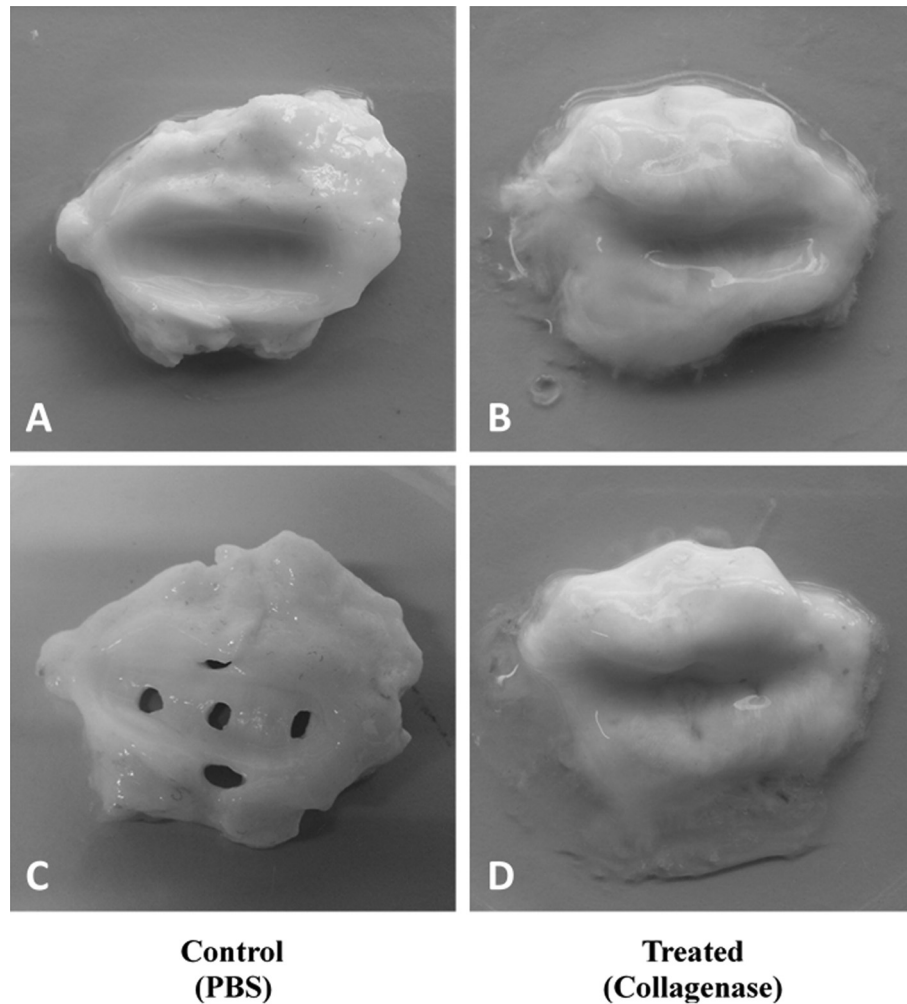
We performed a two-way analysis of variance (ANOVA) to compare the overall effects of collagenase treatment and disc regions on the mechanical and biochemical properties of the associated group. A Bonferroni post-hoc test was used to analyze the regional differences within each group. Also, a paired student's *t*-test was used to analyze the difference between the control (PBS) and collagenase treated samples within a single region of the disc.

All statistical analysis were performed by GraphPad Prism 6.01 (GraphPad Software, La Jolla California) using a significance level of  $P < 0.05$ .

## Results

#### Morphology

The effect of collagenase treatment on gross morphology of the TMJ discs is shown in Fig. 3. The surface layer of all control (PBS)



**Fig. 3.** Effect of collagenase treatment on gross morphology of the TMJ disc. TMJ discs are shown from a superoinferior view after PBS/collagenase incubation (A–B) and after harvesting 4 mm specimens from their five different regions (C–D). The loose gel-like stratum on the surface of treated (collagenase) sample has covered the punched areas on the five regions of the disc (D).

samples were smooth, white and intact [Fig. 3(A)] whereas that of the treated (collagenase) samples seemed to be disrupted and covered with a loose gel-like substance layer [Fig. 3(B)]. After removing samples from the five loaded regions of the disc, the punched locations were clearly visible in the control (PBS) discs [Fig. 3(C)], whereas they were entirely covered by a gel-like substance in the treated (collagenase) discs [Fig. 3(D)].

#### Mechanical compressive properties

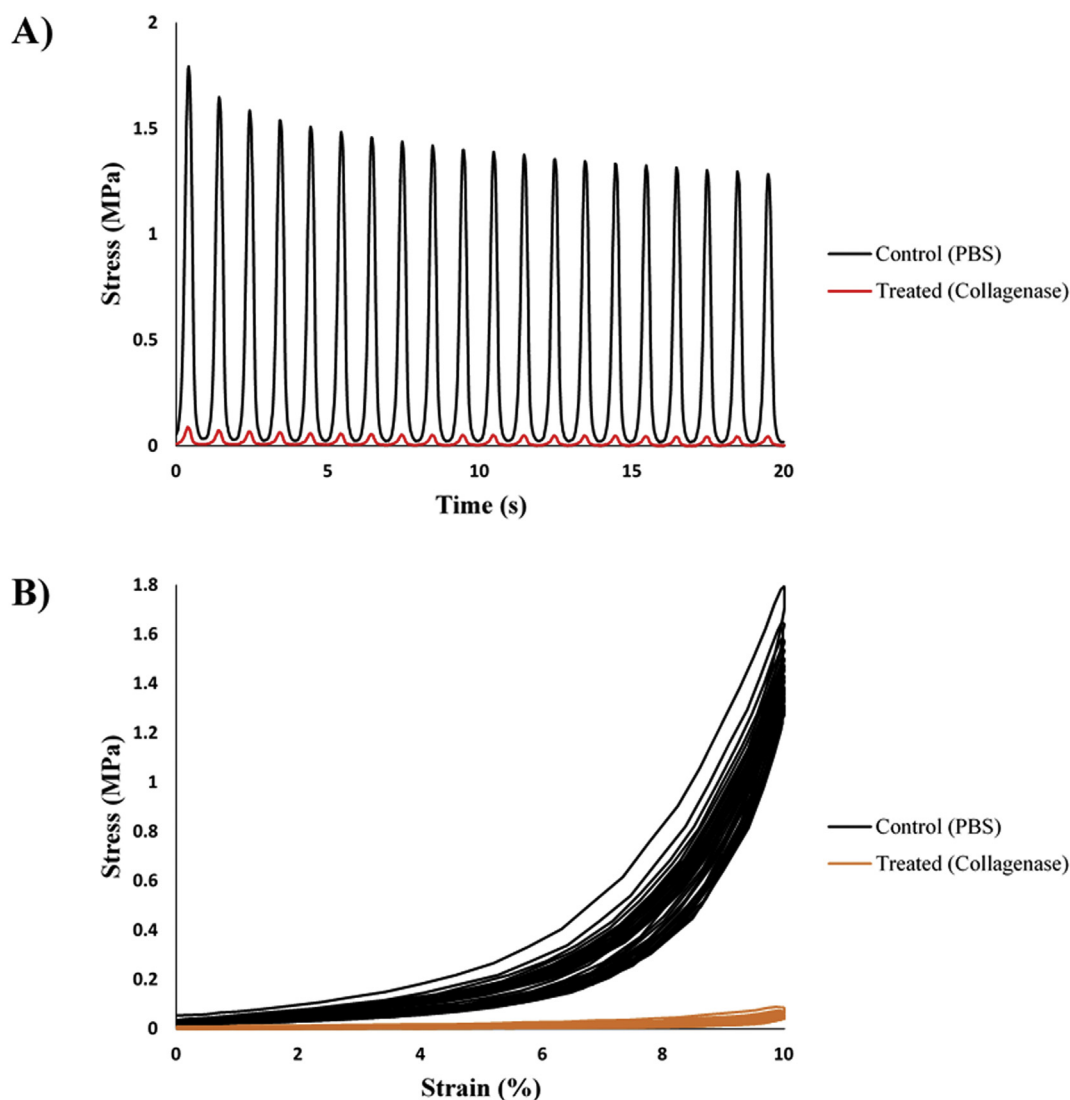
Representative images of the effect of collagenase treatment on stress and hysteresis are shown in Fig. 4. While the strain amplitude was the same during the whole experiment, the stress plateaued off in time [Fig. 4(A)]. Correspondingly, the hysteresis showed the maximum dissipation of energy in the first cycle and then it gradually decreased until the hysteresis loops were almost similar [Fig. 4(B)].

The two-way ANOVA revealed an overall significant effect of both disc region ( $P = 0.002$ ) and collagenase treatment ( $P < 0.001$ ), on the instantaneous modulus across the TMJ discs [Fig. 5(A)]. Application of post-hoc test confirmed the presence of intrinsic heterogeneous properties of the disc by indicating

regional variations of instantaneous modulus in the control (PBS) group. The PB was the stiffest and the IZL was the softest region of the disc. The PB was significantly stiffer than the AB ( $P = 0.037$ ) and IZL ( $P < 0.001$ ). In the intermediate region, IZM was significantly stiffer than IZL ( $P < 0.001$ ). Collagenase treatment resulted in a considerable reduction of instantaneous modulus in all regions. The modulus dropped significantly in IZM (89%,  $P = 0.002$ ), AB (80%,  $P = 0.007$ ), IZC (80%,  $P = 0.007$ ) and PB (67%,  $P = 0.008$ ) while there was no significant decrease in IZL (53%,  $P = 0.119$ ).

The two-way ANOVA indicated that both factors, disc region ( $P = 0.002$ ) and collagenase treatment ( $P < 0.001$ ) had an overall significant impact on energy dissipation capability across the TMJ disc [Fig. 5(B)]. Application of post-hoc test showed regional differences in energy dissipation properties of the control (PBS) group, which is in agreement with variations observed in the corresponding instantaneous modulus data. Most noticeably, PB had the highest energy dissipation capability, which was significantly higher than IZL ( $P = 0.004$ ) and AB ( $P < 0.001$ ). Also, the maximum energy dissipation in the IZM was significantly higher than IZL ( $P = 0.049$ ) and AB ( $P = 0.012$ ). Collagenase treatment significantly reduced the energy dissipation capability of all regions. The reduction was noticeable in the IZM (76%,  $P = 0.0058$ ),





**Fig. 4.** Effect of collagenase treatment on the stress response during cyclic loading. (A) stress vs time and (B) hysteresis in stress-strain response. The control (PBS) data (black) and treated (collagenase) data (red) indicate the significant reduction of stress and energy dissipation capacity of the TMJ disc after the collagenase treatment.

AB (70%,  $P = 0.0091$ ), IZC (69%,  $P = 0.0114$ ), PB (59%,  $P = 0.0008$ ) and IZL (58%,  $P = 0.0275$ ).

#### Biochemical analysis

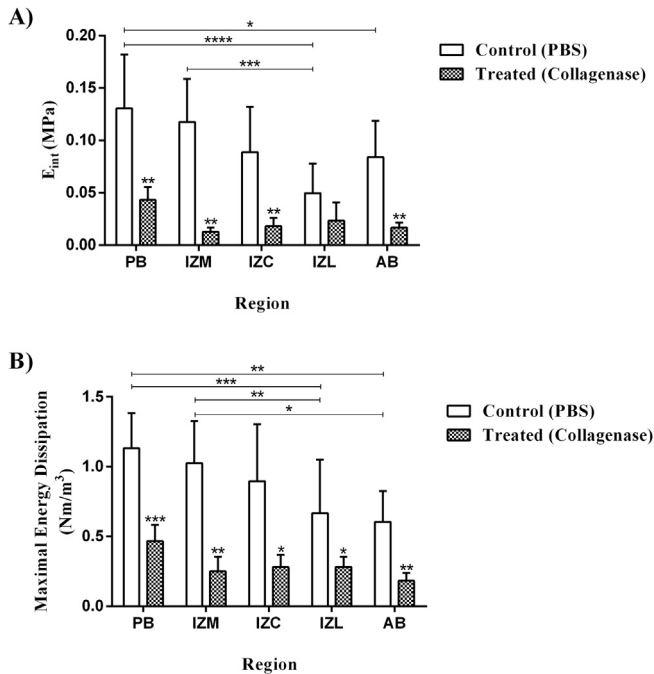
The content of collagen and GAG of the different samples are presented in Fig. 6. The two-way ANOVA indicated an overall significant decrease in collagen content across the TMJ disc due to the collagenase treatment ( $P = 0.0010$ , Fig. 6(A)). However, additional analysis of collagen content between the control (PBS) and treated (collagenase) samples within a single region did not indicate any significant effect of collagenase treatment. More specifically, in the PB and AB, the thickest areas of the disc, collagen was reduced by only 7% ( $P = 0.179$ ) and 10% ( $P = 0.252$ ) respectively. The thinnest areas of the disc, IZC, IZM and IZL, showed relatively larger collagen loss, with 20% ( $P = 0.063$ ), 17% ( $P = 0.142$ ) and 14% ( $P = 0.223$ ) respectively.

The two-way ANOVA revealed an overall significant effect of collagenase treatment ( $P < 0.001$ ) and disc region ( $P = 0.021$ ) on the GAG content across the TMJ disc [Fig. 6(B)]. The post hoc test found regional variations of GAG content in control (PBS) samples with PB

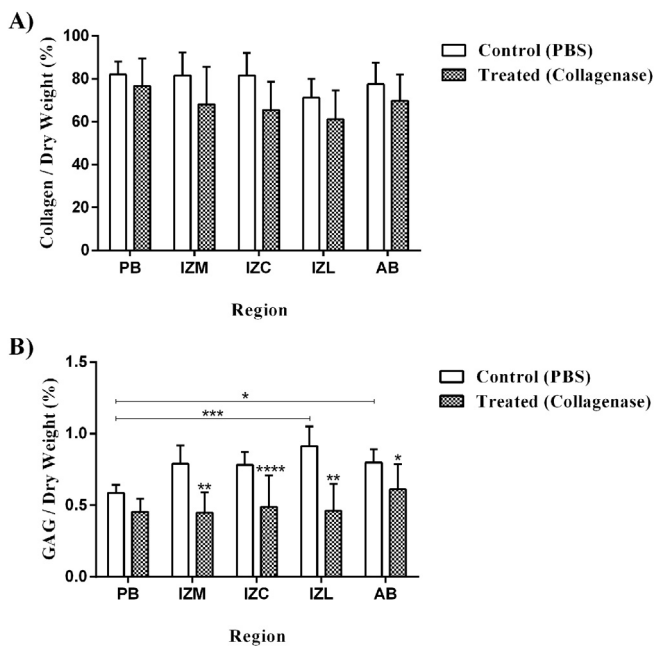
possessing the lowest amount of GAG, which was in turn significantly different with IZL ( $P < 0.001$ ) and AB ( $P = 0.046$ ). Collagenase treatment significantly reduced the GAG content across the thinnest regions as in IZL (37%,  $P = 0.005$ ), IZM (50%,  $P = 0.008$ ) and IZC (43%,  $P < 0.001$ ) whereas thickest areas, AB (23%,  $P = 0.045$ ) and PB (24%,  $P = 0.113$ ) were less affected.

#### PLM

Representative PLM images of control (PBS) and treated (collagenase) samples are depicted in Fig. 7 and Fig. 8. The superoinferior PLM images revealed a ring-like arrangement of fibrillar collagen in the peripheral regions and the anteroposteriorly aligned fibers through the intermediate zone (IZL, IZM and IZC). Collagen fibers in the control (PBS) samples were well aligned, organized in a parallel arrangement and showing regularity (Figs. 7 and 8). The collagenase treatment perturbed the structural integrity of the collagenous network resulting in an indistinct fiber arrangement (Figs. 7 and 8).



**Fig. 5.** Effect of collagenase treatment on mechanical compressive properties of the TMJ discs. A: Collagenase treatment had a substantial effect on the instantaneous modulus of TMJ discs in all regions. The regional variation was observed for control (PBS) samples. B: Both collagenase treatment and disc region factors had a significant effect on energy dissipation capabilities of the TMJ discs. Collagenase treatment substantially reduced the maximum energy dissipation capability within all regions, which shows similar tendency observed for the instantaneous modulus data. All data are presented as mean  $\pm$  95% confidence interval. \*indicates significant difference ( $n = 5$ ,  $P < 0.05$ ).



**Fig. 6.** Effect of collagenase treatment on collagen and GAG content of TMJ discs. (A) Approximately 80% of the dry weight of TMJ disc is composed of collagen fibers. Despite overall decrease of collagen content across the TMJ disc, the collagenase treatment did not induce significant collagen loss within single regions of the treated (collagenase) sample. (B) GAG only consists less than 1% of TMJ disc's dry weight. Collagenase treatment induced GAG reduction in all regions. Also as a heterogeneous tissue, control (PBS) samples showed GAG regional variation. All data are presented as mean  $\pm$  95% confidence interval. \*indicates significant difference ( $n = 5$ ,  $P < 0.05$ ).

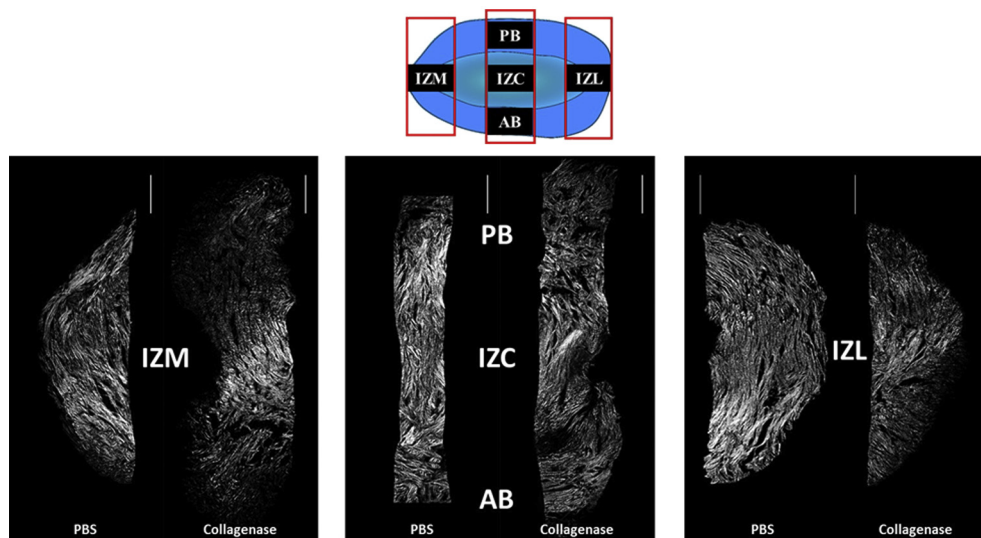
## Discussion

The TMJ disc is a highly strained cartilaginous construct with a functional collagenous structure. Distortion of the disc architecture by degeneration strongly affects functionality and results in pain in a large portion of the population<sup>28</sup>. The aim of the present study was to analyze the contribution of the highly organized collagen fibers to the load bearing capacity in the articular disc of the TMJ. Therefore, we perturbed its structural collagenous network by collagenase treatment and performed mechanical tests before and after. The collagenase treatment induced a derangement of fibers altering the biochemical composition (collagen and GAG) of the TMJ disc to a certain extent. Yet, its stiffness was greatly affected, and herewith its load bearing capacity. Furthermore, following the collagenase treatment, it appeared that TMJ disc was no longer mechanically and biochemically heterogeneous.

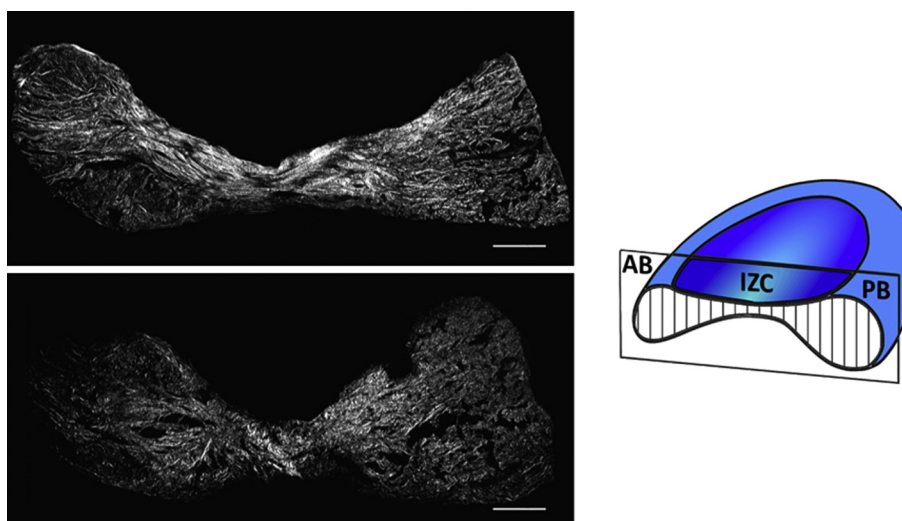
The heterogeneous nature of the undisturbed TMJ disc was manifested in its regional variation of mechanical and biochemical properties. In the control group, the PB and IZM showed the highest compressive stiffness in contrast to the IZL. This is in agreement with Allen and Athanasiou<sup>17</sup> and Kim *et al.*<sup>8</sup>. However, Beek *et al.*<sup>14</sup> and, Nickel and McLachlan<sup>29</sup> found the IZC as the stiffest region under compression. This contradiction may be due to the different methodologies and materials used in their studies. For example, Beek *et al.*<sup>14</sup> used aged human samples and applied large strains (20–40%), and did not consider mediolateral differences. Additionally, a similar regional variation as in compressive stiffness was observed in maximal energy dissipation of the control (PBS) samples. This mechanical characteristic facilitates the TMJ disc with stress absorbing capabilities to prevent the accumulation of excessive strain energy leading to associated potential damage<sup>30,31</sup>.

Following the collagenase treatment, the compressive moduli of the disc were reduced by approximately 50–90%, depending on the regions among which the IZM showed the largest and IZL the smallest reduction. A similar reduction pattern was observed for maximal energy dissipation following the treatment, marking the weakened shock absorbing ability in the TMJ disc. Due to the non-uniform reduction of the mechanical parameters with respect to the various regions it appeared that the original heterogeneity of the disc had disappeared almost completely.

The contribution of collagen fibers to the compressive stiffness of the TMJ disc can be explained from different perspectives. Beek *et al.*<sup>14</sup> suggested that when the disc is compressively loaded, the anteroposteriorly oriented collagen fibers direct the interstitial fluid flow towards the anterior and posterior bands where it is arrested by mediolaterally oriented fibers. Although we found PB as the stiffest region of the untreated TMJ disc under compression, the mediolaterally oriented fibers in that region can be expected to serve the same general function as stated by Beek *et al.*<sup>14</sup>. Therefore, the regional arrangement of collagen fibers may act as a pathway for compression-induced fluid flow in an effectively functional way herewith distributing force and reducing stress concentration on the directly loaded region. This is in agreement with earlier findings, showing that diffusion along the fiber orientation was significantly faster than transverse to it<sup>32</sup>. Likely, the collagenase treated samples lost much of their fluid diffusion directionality leading to a reduction in their energy dissipation capability. However we cannot directly correlate the weakened mechanical properties of the treated (collagenase) samples to their altered fluid flow pattern resulted by derangement of collagen fibers. Further studies are needed to investigate this in detail. Also, the straightened collagen fibers in surface layer of cartilage have been demonstrated to act like a trampoline under compression, spreading the direct load and consequently reducing the applied stress<sup>23,33–35</sup>.



**Fig. 7.** Effect of collagenase treatment on the integrity of the collagenous network. PLM images of representative regions of TMJ disc seen from above. The region-dependent collagen fiber alignment with its typical ring-like structure is clearly shown across all regions. Comparison of control (PBS) samples with treated (collagenase) samples in each region shows the lack of structural integrity in the collagenous network of the latter group. Scale bar: 2 mm.



**Fig. 8.** Effect of collagenase treatment on the integrity of the collagenous network. PLM images of representative regions of TMJ disc seen in the sagittal plane. The collagen fibers are aligned anteroposteriorly perpendicular to the mediolaterally oriented fibers in the PB and AB (not shown in this view). Comparison of control (PBS) samples with treated (collagenase) samples shows the lack of structural integrity in the collagenous network of the latter group. Scale bar: 2 mm.

Collagen constituted about 80% and GAG approximately 1% of the untreated TMJ discs, which is in line with previous studies<sup>10,22</sup>. The PB and IZM had the highest collagen content, although these differences were not significant. Remarkably, they were also the regions that showed the largest reduction in mechanical properties after collagenase treatment. However, following the collagenase treatment, the reduction of collagen content was much less than the reduction in stiffness. This is likely due to the selected combination of treatment time and collagenase concentration, which led to only the perturbation of the collagenous network without a significant loss of collagen<sup>36</sup>. This implies that the contribution of collagen content as such to the mechanical stiffness is limited.

The difference in GAG concentration in the untreated group was most pronounced between PB and IZL, which had the lowest and highest GAG content respectively. In contrast to the limited reduction of collagen content, GAG content was significantly reduced following the collagenase treatment. PGs loss is attributed

to the disintegration of collagenous network as it releases PGs entrapped within the cartilage matrix<sup>37</sup>. The relatively minor influence of reduction of GAG on the mechanical behavior can be explained by their very small amount in untreated TMJ discs to begin with. This is in line with Willard *et al.*<sup>20</sup> who showed that 96% removal of GAG from all regions of the TMJ disc did not influence its instantaneous compressive modulus. Therefore, in this study, the observed alteration of mechanical properties in the TMJ disc is more likely related to its perturbed collagenous network than the alteration of its biochemical composition. This is in agreement with Detamore *et al.*<sup>38</sup>, who described a lack of straightforward correlation between mechanical compressive properties and biochemical content in TMJ disc.

The PLM images confirmed the loss of the structural organization of the collagenous network after collagenase treatment of the TMJ disc. This was illustrated by a haphazard arrangement of fibers, loss of fibrillar interconnectedness and reduced collagen fiber

alignment in the treated samples. A disorganized collagenous network in enzymatically or naturally degenerated cartilage has been observed<sup>12,37,39–41</sup>. For example, Thambyah *et al.*<sup>41</sup> observed fibrillar aggregation and loss of transverse fibrillar interconnectivity in the matrix of degenerated cartilages. Morphologically, the collagenase treatment disrupted the superficial surface of the treated samples and produced a loose gel-like layer covering their surface. Wang *et al.*<sup>40</sup> observed a disorganized superficial structure with irregular collagen-free lamina in inflamed TMJ discs with scanning electron microscopy and transmission electron microscopy. Since an intact surface of cartilage may attenuate the applied stress, its damage may have consequences for the mechanical behavior of the complete structure.

The region-dependent mechanical properties of the TMJ disc supposedly play a role in jaw kinematics by defining an articulation path along its anteroposterior axis<sup>17,23,42</sup>. Considering the small distances separating different regions of the disc, it is crucial to note that even minor deviation of the disc from the articulation path as in the case of internal derangement of the disc, may lead to adverse biomechanical loading conditions<sup>8</sup>. Consequently mechanical overloading of the cartilage results in more catabolic cytokine production and enhanced matrix degradation<sup>5,15,43</sup>. Fragmentation of collagen fibrils followed by disintegration of the collagenous network are the earliest signs of OA<sup>5,15,44</sup>. The clinical importance of early recognition of any malfunctionality related to the disc such as internal degradation, underscore the importance of an appropriate collagen arrangement and structural integrity of TMJ matrix. Enzymatic degradation in combination with mechanical loading has been commonly applied in the literature to mimic the *in vitro* OA in cartilage<sup>20,36,45,46</sup>. Collagenase digestion thus can induce disintegration of the collagenous network, which initiates or enhances the degenerative process in cartilage<sup>37</sup>.

Overall, the contribution of the collagen fibers and their region-specific arrangement appeared to play a vital role in stabilizing the TMJ disc under compression. Our study shows that even mild disruption of the collagenous network in the TMJ disc, with marginal alteration of collagen density can produce a dramatically affects its mechanical compressive properties. It has been a challenging goal for tissue engineers to construct a TMJ disc with a quantitatively dominant and structurally organized collagenous part. Achieving this goal demands an in-depth knowledge of the structure-function relationship between the components of the extracellular matrix of TMJ disc. Current data suggest tissue engineers to characterize the TMJ disc compressive behavior with a prior focus on the role of its structural integrity of collagenous network. Quantitative measurement of collagen fiber alignments and collagen cross-links could further enhance our understanding of the fibrillar interconnectivity and the reinforcing role of collagen fibers in the TMJ disc.

### Author contributions

S.F., S.G., V.E., T.H.S. and J.H.K. designed the experiments; S.F. and S.G. conducted the experiments; S.F. analyzed the data and wrote the manuscript text. All authors reviewed the manuscript.

### Conflict of interest

The authors have declared that no conflict of interest exist.

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